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Isolation of RNA Polymerase from *Clostridium difficile* and Characterization of Glutamate Dehydrogenase and rRNA Gene Promoters In Vitro and In Vivo

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Clostridium difficile **is the primary causative agent of antibiotic-associated diarrheal disease. To facilitate molecular genetic analysis of gene expression in this organism, methods were developed to study transcriptional regulation in vitro and in vivo. That is, *C. difficile* RNA polymerase was partially purified and shown to bind to and initiate transcription in vitro from bona fide *C. difficile* promoters for rRNA and glutamate dehydrogenase genes. In addition, primer extension analyses and a β-glucuronidase reporter system were used to quantitate transcription from these promoters in vivo. With these tools in hand, it is now possible to characterize the behavior of any *C. difficile* gene in vivo and to study the regulation of its expression in detail.**

Clostridium difficile is the major organism implicated in antibiotic-associated colitis and its potentially fatal consequence, pseudomembranous colitis (11, 19, 20). While much is known about the biochemical activities of two large toxin proteins that are thought to be the principal factors that cause disease (2, 16, 17, 25, 31, 32), it has proven to be unusually difficult to obtain basic information about the physiology and regulation of gene expression in this organism. Two factors have contributed to this disparity. First, no widely applicable methods of genetic exchange or directed mutagenesis have been successfully applied to *C. difficile*. As a consequence, little is known about the roles of individual genes in growth or pathogenesis. Second, information about fundamental mechanisms of gene regulation used under various environmental conditions is very limited.

The best-studied mechanism of gene regulation in *C. difficile* controls the synthesis of the toxin proteins, the two large glycosyl transferases that modify members of the host cell Rho protein family and thereby disrupt the actin cytoskeleton (16, 17, 32). The toxin genes *tcdA* and *tcdB* are carried in a 19-kb pathogenicity locus (5, 7, 12). The same pathogenicity locus encodes TcdR (previously known as TxeR or TcdD) (28), a protein that acts as an alternative sigma factor of RNA polymerase to activate transcription of *tcdA* and *tcdB* (23). Synthesis of TcdR is regulated by the growth state and the availability of certain nutrients (24). As a result, toxin synthesis also responds to environmental signals (9, 18, 23, 24).

To broaden our understanding of the basic physiology of *C. difficile* and to permit detailed analyses of gene expression, we have developed and adapted genetic and molecular tools that permit quantitation of gene expression both in whole cells and in a purified in vitro transcription system. These tools were used here to study fundamental aspects of the expression of rRNA genes and the gene for glutamate dehydrogenase. Similar preparations of RNA polymerase were shown previously to bind to the *gdh* promoter but to lack TcdR (23, 24). As a result, this form of RNA polymerase does not recognize the *tcdA*, *tcdB*, or *tcdR* promoter (23, 24).

MATERIALS AND METHODS

Bacterial strains and growth media. *C. difficile* strain VPI 10463 (12) was used for RNA polymerase purification. *C. difficile* strain CD630 (15) chromosomal DNA was used as the source for PCR amplification of the *rrn* promoters. *Clostridium perfringens* strain SM101 (34) was used as the heterologous host for expression of reporter fusions. *C. difficile* and *C. perfringens* strains were grown in an anaerobic chamber in tryptone-yeast extract (TY) medium or TY medium supplemented with 1% glucose (TYG), as described previously (9). For plasmid-carrying strains of *C. perfringens*, chloramphenicol was added to a final concentration of 20 μg/ml. All routine plasmid constructions and cloning in *Escherichia coli* were performed according to standard procedures (29).

Purification of *C. difficile* RNA polymerase. Cells from an 8-liter culture of *C. difficile* VPI 10463, grown anaerobically to early stationary phase in TY medium, were harvested by centrifugation at 11,000 × *g*, washed once in cold buffer A (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1 mM dithiothreitol [DTT], 2 mM phenylmethylsulfonyl fluoride [PMSF], 10% glycerol), and suspended in 30 ml cold buffer A containing 100 mM KCl. The cells were broken by three passages through a French pressure cell at 10,000 lb/in^2, followed by sonication in three 30-s pulses. The resulting cell lysate was clarified by centrifugation at 27,000 × *g*, and the supernatant fluid was subjected to differential ammonium sulfate precipitation, leading to the collection of proteins precipitated between 30% and 85% saturation. The resulting protein pellet was resuspended in and dialyzed against buffer B (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1 mM DTT, 2 mM PMSF, 20% glycerol) before being loaded onto a 30-ml column of DEAE-Sephacel (Sigma) prepared according to the manufacturer's instructions. Proteins were eluted with buffer B containing stepwise 0.1 M increments of KCl (0.2 M to 0.8 M). The bulk of the RNA polymerase activity (assayed as described below) eluted at 0.5 M KCl. This fraction (~30 ml) was dialyzed against buffer B, and the glycerol content was adjusted to 50%. Ten milliliters of the dialyzed fraction was then loaded onto a 10-ml column of cellulose phosphate (Whatman) prepared according to the manufacturer's instructions and equilibrated with buffer C (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1 mM DTT, 2 mM PMSF, 50% glycerol) containing 0.1 M KCl. RNA polymerase was eluted by buffer C containing stepwise 0.1 M increments of KCl (0.2 M to 0.8 M), followed by 1.0 M and 2.0 M KCl. The phosphocellulose column fractions having RNA poly-

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merase activity were diluted with buffer D (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1 mM DTT, 2 mM PMSF) and concentrated 100-fold using a 10,000-molecular-weight-cutoff centrifugal filtration column (Millipore). Glycerol was then added to a final concentration of 50%, and RNA polymerase was stored at −80°C in multiple aliquots. The protein concentrations of the various fractions were determined by the method of Bradford (4). The purity of the proteins during various stages of purification was assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and staining with either Coomassie blue (29) or a silver-staining kit (Bio-Rad) according to the manufacturer's instructions.

Other sources of RNA polymerase. *E. coli* RNA polymerase holoenzyme and core enzyme forms were purchased from Epicentre. *Bacillus subtilis* RNA polymerase holoenzyme was prepared by F. W. Whipple (33).

Cloning of the *gdh* and *rrn* promoters from *C. difficile*. A plasmid containing the *C. difficile* glutamate dehydrogenase (*gdh*) promoter was provided by Lisa Barosso (Virginia Polytechnic Institute). The *gdh* promoter region was amplified by PCR using the oligonucleotide pair ONM31 (5′-CGGCTGCAGGGTTTTAGCTGGGATATCGGC-3′) and ONM32 (5′-CGCTCTAGACATCTCGAAGACATTTACATC-3′), corresponding to positions −831 to +33 with respect to the translational start point, or the oligonucleotide pair ONM31 and ONM33 (5′-CGCTCTAGACATACCTAATTTATCACATGC-3′), corresponding to positions −831 to +75 relative to the translation start point. Embedded PstI and XbaI restriction sites (underlined) allowed cloning in the corresponding sites of the vector pSM151 (34) (a derivative of plasmid pBS+ [Stratagene] carrying the transcription termination signals of the *C. perfringens cpe* gene), resulting in plasmids pTUM198 and pTUM199, respectively.

The *rrnC* and *rrnE* promoters were amplified by PCR from the *C. difficile* CD630 genome using the oligonucleotide pair ONM56 (5′-CGCCTGCAGCTAATGCCAATATGTTTGTCTC-3′) and ONM57 (5′-CGCGGATCCGGCACGCCGCCAGCGTTCATC-3′) for *rrnC* and the oligonucleotide pair ONM58 (5′-CGCCTGCAGGTGTTTTTGGTTGAGCAATATATG-3′) and ONM57 for *rrnE* with engineered PstI and BamHI sites (underlined) and cloned in pSM151 at the corresponding restriction sites, resulting in plasmids pTUM549 and pTUM550.

In vitro transcription reactions. To monitor RNA polymerase activity during purification, in vitro transcription reactions were carried out in a 100-μl volume containing 40 mM Tris-HCl, pH 8.0; 10 mM $MgCl_2$; 0.1 mM EDTA; 1 mM DTT (added freshly); 0.05 M KCl; 0.1 mg bovine serum albumin per ml; 5% (vol/vol) glycerol; 1 mM $MnCl_2$; 200 μM each of ATP, GTP, and CTP; 50 μM unlabeled UTP; 2.5 μCi [α-^{32}P]UTP (600 Ci/mmol; NEN); 2 μg of poly(dA-dT) template; and various amounts of RNA polymerase. The reaction mixtures were mixed and incubated at 37°C for 30 min, after which the synthesized RNA was precipitated by the addition of 1.5 ml of cold 5% trichloroacetic acid and incubation on ice for 10 min. The precipitates were collected on nitrocellulose filters under a vacuum, dried with a heat lamp, suspended in Ready Safe scintillant (Beckman), and analyzed with a scintillation counter.

In vitro transcription reactions using nonsynthetic templates were carried out similarly to the reactions described above except that the volume was 10 μl and contained 2 units of RNasin (Promega) and 2 μg of linearized plasmid DNA or 2 μg of closed circular plasmid DNA instead of poly(dA-dT). Plasmids pTUM198 and pTUM199, digested with XbaI, were used as templates for *gdh* runoff transcription. Plasmids pTUM549 and pTUM550 that were digested with BamHI were used as templates for in vitro runoff transcription from the *rrnC* and *rrnE* promoters, respectively. When closed circular forms of the same plasmid DNAs were used, transcription stopped at termination sites encoded within the DNA. The reactions were stopped by adding 5 μl of formamide buffer (29) and heating at 80°C for 10 min. Five-microliter samples were loaded directly onto gels containing 5% polyacrylamide and 8 M urea. Following electrophoresis, the gels were transferred to filter paper, dried, and exposed either to a phosphorimager screen or to X-ray film.

Gel retardation experiments. Gel mobility shift assays were carried out as previously described (23). Fragments of 330, 428, and 488 bp, corresponding to positions −324 to +6, −372 to +56, and −431 to +57, respectively, with respect to the translational start codon of the *gdh* gene and to the predicted 5′ end of mature 16S RNA for the *rrnC* and *rrnE* genes, were amplified by PCR from pTUM199 (for the *gdh* promoter) (23) or pTUM549 or pTUM550 (for the *rrnC* and *rrnE* fragments) and then end labeled with T4 polynucleotide kinase (U.S. Biochemicals, Cleveland, Ohio) and [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham). The labeled fragments (0.2 nM) were incubated for 60 min at room temperature in 10 μl of glutamate buffer containing 100 nM RNA polymerase. Four microliters of a heparin dye solution (150 μg of heparin per ml, 0.1% bromophenol blue, 50% sucrose) in glutamate buffer was added, and the mixture was loaded during electrophoresis onto a 4.5% polyacrylamide gel prepared in Tris-borate-EDTA buffer. After electrophoresis (2 h at 13 V/cm), the gel was dried, transferred onto filter paper, and analyzed by autoradiography. Competition studies were carried out with a preincubation step of 10 min in an excess of unlabeled nonspecific competitor [1 mg poly(dI-dC)] or homologous DNA (plasmid DNA corresponding to the *rrnC* and *rrnE* promoters) before the addition of labeled probes.

Primer extension reactions. Total RNA was isolated from 10-ml cultures of *C. difficile* strains VPI 10463 and CD630, grown anaerobically in TY medium to mid-exponential or early stationary phase, using the QIAGEN RNeasy kit according to the manufacturer's instructions. RNA concentrations were determined spectrophotometrically, and purity was assessed by agarose gel electrophoresis followed by ethidium bromide staining.

The primers ONM60 (5′-CATACCTAATTTATCACATGC-3′), corresponding to positions +55 to +75 with respect to the start codon of the *gdh* gene, and ONM61 (5′-GGCACGCCGCCAGCGTTCATC-3′), corresponding to positions +26 to +46 with respect to the expected 5′ end of the mature 16S rRNA gene, were 5′-end labeled by incubation with T4 polynucleotide kinase and [γ-^{32}P]ATP for 1 h at 37°C. The labeled primers were purified using the QIAGEN nucleotide removal kit and quantified by scintillation counting. For primer extension reactions, 5 to 10 μg of total RNA was mixed with the radiolabeled primers and extended using Superscript II RNase H^- reverse transcriptase (Gibco BRL) according to the manufacturer's instructions. The extension products were mixed with formamide loading buffer (29), denatured by heating to 80°C, and subjected to electrophoresis on a 5% polyacrylamide–8 M urea sequencing gel along with DNA-sequencing reactions performed on plasmid pTUM199 (*gdh* promoter) or pTUM550 (*rrnE* promoter) using primer ONM60 or ONM61, respectively, [α-^{35}S]dATP, and Sequenase DNA polymerase (USB) according to standard procedures (29). The gels were vacuum dried and exposed to a phosphorimager screen.

Construction of a GusA reporter fusion. In order to construct a P*gdh-gusA* fusion, the *gdh* promoter DNA (i.e., the PCR product generated by the primer pair ONM31 and ONM33) was cloned as an EcoRI-XbaI fragment in the corresponding sites of the *gusA* fusion plasmid pTUM177 (23). The resulting plasmid, pTUM270, was introduced into *C. perfringens* SM101 by electroporation (34), and β-glucuronidase activity was measured in cells grown anaerobically to mid-exponential phase in either TY or TYG medium as described previously (9).

RESULTS

***C. difficile* RNA polymerase isolation.** We sought to purify *C. difficile* RNA polymerase in a form that would faithfully recognize bona fide *C. difficile* promoters and aid in the study of the effects of regulatory proteins on gene transcription in in vitro transcription assays. As described in Materials and Methods, RNA polymerase activity in a crude extract was precipitated by the addition of ammonium sulfate to 85% saturation. The RNA polymerase activity in the redissolved and dialyzed precipitate failed to bind to heparin-Sepharose, single-stranded DNA-agarose, DNA-cellulose, or BioRex-70 columns despite the fact that such columns are routinely used for purification of RNA polymerase from other organisms. *C. difficile* RNA polymerase did, however, bind to a DEAE-Sephacel column and was eluted at 0.5 M KCl, giving substantial purification. *C. difficile* RNA polymerase was subsequently bound to and eluted from a phosphocellulose column. The phosphocellulose column chromatography was carried out in buffer containing 50% glycerol to prevent the separation of sigma factors and core RNA polymerase (6). Even though a significant amount of the RNA polymerase activity was eluted from the column during the wash step (0.1 M KCl), we were able to obtain adequate supplies of partially purified RNA polymerase by elution with 0.4 M KCl. The partially purified *C. difficile* RNA polymerase preparation was subjected to electrophoresis on an SDS-containing 4 to 20% polyacrylamide gradient gel. As shown in Fig. 1, two bands with mobilities expected for the β and β′ subunits (predicted masses of ~130

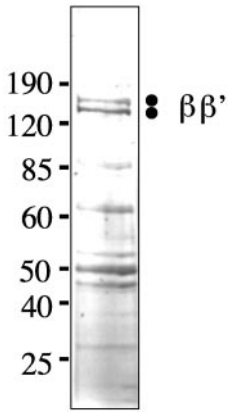

FIG. 1. SDS-PAGE analysis of RNA polymerase from *C. difficile*. RNA polymerase was isolated from *C. difficile* VPI 10463 cells. A partially purified extract was loaded onto a phosphocellulose column, and proteins that eluted with 0.4 M KCl were analyzed on a 4 to 20% gradient SDS-PAGE gel. The protein bands were visualized by silver staining. The putative β and β′ subunits of *C. difficile* RNA polymerase are indicated by two dots.

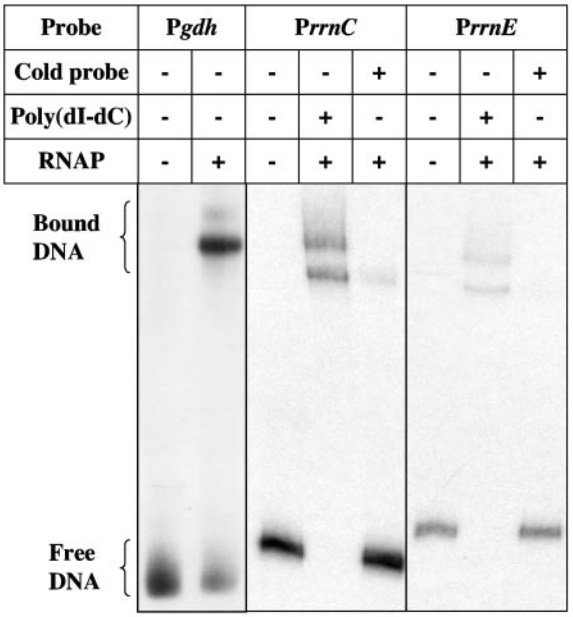

FIG. 2. Gel mobility retardation of *gdh* and *rrn* promoters by *C. difficile* RNA polymerase. DNA fragments containing the *C. difficile* glutamate dehydrogenase (P*gdh*) and the *C. difficile rrnC* and *rrnE* operon promoter regions (P*rrnC* and P*rrnE*) were incubated with 100 nM *C. difficile* RNA polymerase. For the *rrn* promoters, competitions were carried out with nonspecific, unlabeled DNA [poly(dI-dC)] or specific, unlabeled plasmid DNAs (pTUM549 [*rrnC*] and pTUM550 [*rrnE*]) added to the binding reactions.

and ~140 kDa, respectively) and comparable to the corresponding β and β′ subunits of *E. coli* and *B. subtilis* RNA polymerases were observed. Additional polypeptides with mobilities consistent with the sizes of *C. difficile* α and σ^A subunits of RNA polymerase (predicted masses of 35 and 44 kDa, respectively) were also observed. The identities of the *C. difficile* α and σ^A bands cannot be assigned with confidence, however, because these subunits sometimes have aberrant mobilities in SDS-PAGE. *B. subtilis* σ^A (mass, 43 kDa) has the mobility of a 57-kDa polypeptide (21), and *E. coli* σ^{70} (mass, 70 kDa) migrates at the position expected for a polypeptide of 85 to 95 kDa (22).

***C. difficile* RNA polymerase binds to the promoter regions of the *gdh*, *rrnC*, and *rrnE* genes.** To test whether the partially purified *C. difficile* RNA polymerase had the function expected for the major vegetative (σ^A-containing) holoenzyme form, we performed gel mobility shift assays with DNA fragments likely to contain the promoter regions of the *C. difficile gdh*, *rrnC*, and *rrnE* genes. (Note that we have assigned names to the 11 *C. difficile rrn* operons based on their order in the total genome sequence [http://www.sanger.ac.uk/Projects/C_difficile/].) Our results showed that the purified RNA polymerase was able to bind to and shift the mobility of all these DNAs (Fig. 2). The specificity of the binding of *C. difficile* RNA polymerase to both the *rrnC* and *rrnE* promoters was demonstrated by the fact that this binding was unaffected by the presence of poly(dI-dC), a nonspecific DNA competitor, but was abolished when an excess of the corresponding unlabeled promoter DNA was included in the reaction mixture (Fig. 2).

***C. difficile* RNA polymerase activates transcription in vitro from the *gdh*, *rrnC*, and *rrnE* promoters.** We performed in vitro runoff transcription assays with *C. difficile gdh*, *rrnC*, and *rrnE* promoter-containing DNAs to determine whether the interaction of *C. difficile* RNA polymerase with these promoters leads to the activation of transcription. Indeed, the purified *C. difficile* RNA polymerase was able to activate transcription from the *gdh* (Fig. 3A) and *rrn* (Fig. 3B) promoters in vitro. As shown in Fig. 3A, runoff transcripts produced from *gdh* templates cleaved at restriction sites located 42 bp apart yielded transcripts that differed in length by ~40 nucleotides (nt). Similarly, closed circular templates in which the transcription termination sites were located 42 bp apart gave terminated transcripts of the appropriate sizes. For the *rrn* promoters, both linear and circular templates yielded transcripts of the sizes expected based on sequence analysis of the likely transcription start point (see below). Transcription products of the same sizes were produced by the *C. difficile*, *E. coli*, and *B. subtilis* RNA polymerases (Fig. 3), indicating that the three enzymes recognize similar promoter sites in the template DNAs.

Mapping the transcription start points for *gdh*, *rrnC*, and *rrnE* genes in *C. difficile*. In order to compare the transcription start sites in vitro with those used in living cells, primer extension analysis was performed on the *C. difficile gdh* mRNA and *rrn* transcripts. As shown in Fig. 4A, a cDNA of 146 nt was synthesized in the case of the *gdh* gene. This cDNA (Fig. 4B, lanes 3 and 4) had the same electrophoretic mobility as the cDNA derived from primer extension of the RNA product of in vitro transcription (Fig. 4B, lane 2). The in vitro-synthesized RNA itself had a slightly lower mobility (Fig. 4B, lane 1). This discrepancy was expected, since RNA has ~5% lower mobility than does cDNA of the same length (29).

A similar comparison was attempted with the *rrn* promoters in vivo. However, since *C. difficile* has 11 nearly identical copies of the *rrn* operons (data not shown), and because *rrn* transcripts undergo a series of rapid endonucleolytic processing

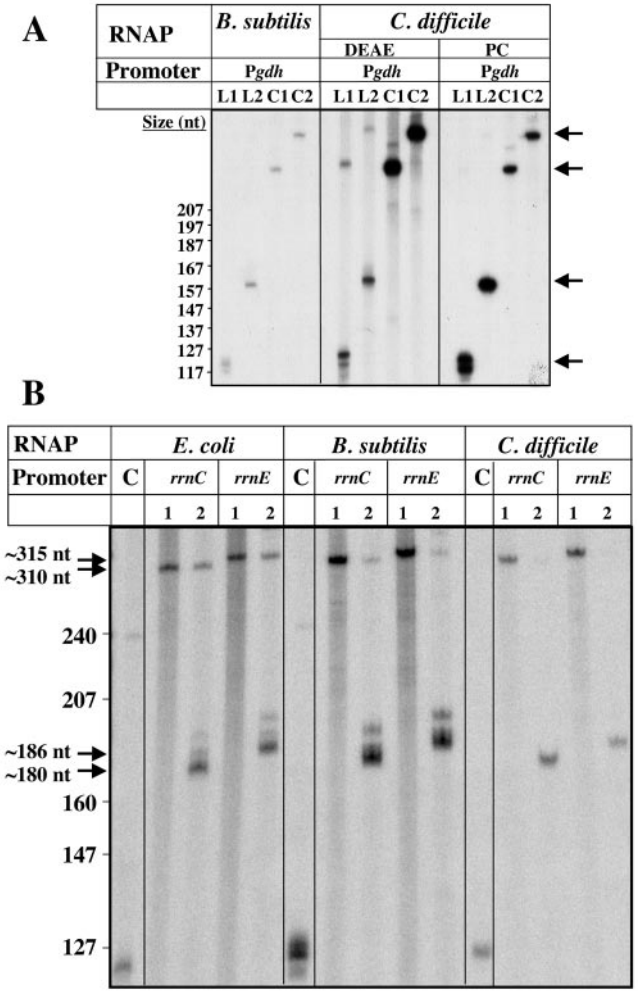

FIG. 3. In vitro runoff transcription of *gdh* and *rrn* promoters by *E. coli*, *B. subtilis*, or *C. difficile* RNA polymerase. (A) In vitro transcription reactions with active fractions of *C. difficile* RNA polymerase (RNAP) from DEAE-Sephacel and phosphocellulose (PC) columns resulted in specific runoff transcripts from the *C. difficile* glutamate dehydrogenase promoter (P*gdh*) DNA. The same promoter was also recognized by a purified *B. subtilis* RNA polymerase preparation. L1 and L2 (pTUM198 and pTUM199, respectively) refer to linearized plasmids carrying different lengths of *C. difficile* DNA downstream of the *gdh* promoter, whereas C1 and C2 refer to circular forms of the same plasmids. In pTUM198 and pTUM199, the *gdh* promoter-containing fragments were cloned upstream of a factor-independent transcription terminator. (B) In vitro transcription reactions with *E. coli*, *B. subtilis*, and purified *C. difficile* RNA polymerase resulted in specific transcripts from the *C. difficile rrnC* and *rrnE* operon promoters. Plasmids containing versions of the rRNA gene promoters with different 3′ ends were used as circular plasmid templates (lane 1) or as linearized templates (lane 2). The uppermost band in lane 2 is presumed to derive from transcription of uncut plasmid molecules. The control transcription reactions (lane C) refer to linearized plasmid pTUM198 carrying the *gdh* promoter (the same as in lane L1 described above).

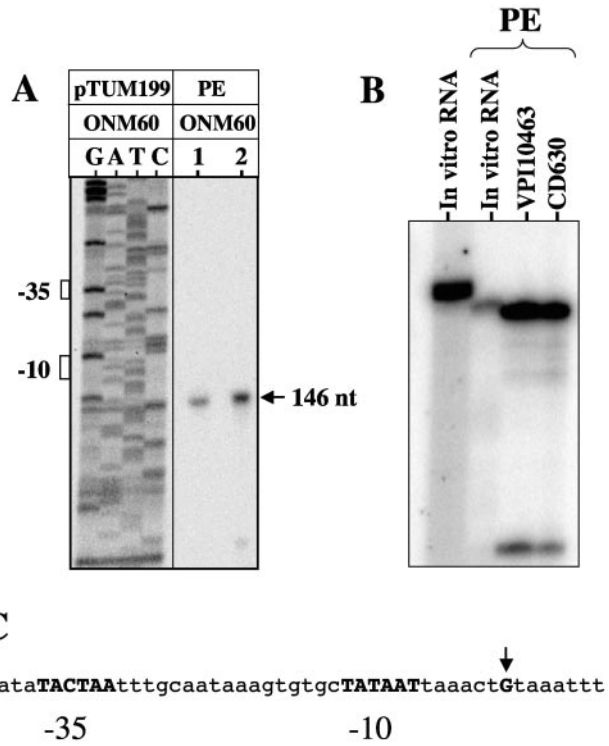

FIG. 4. Determination of the transcription start site of the *gdh* gene. (A) Total RNA from *C. difficile* VPI 10463 and CD630 was used as a template to extend primer ONM60 using reverse transcriptase. The reverse-transcribed products, corresponding to *C. difficile gdh* mRNA from VPI 10463 (lane 1) and CD630 (lane 2), were separated by electrophoresis alongside DNA-sequencing reaction products (shown on the left) generated using plasmid pTUM199 (harboring the *C. difficile gdh* gene) as a template and primer ONM60. (B) In vitro transcription products of the *gdh* gene generated using XbaI-digested plasmid pTUM199 as a template were separated by electrophoresis alongside the primer extension (PE) product generated from the in vitro-transcribed mRNA using primer ONM60 and compared to the primer extension products generated using total RNA from *C. difficile* strains VPI 10463 and CD630. (C) DNA sequence corresponding to the *gdh* transcription start site (indicated by an arrow) and the location of the hexanucleotide −10 and −35 sequences (uppercase letters).

events in vivo (1), the detection of transcription of a particular *rrn* operon in vivo is difficult. Thus, while the in vitro runoff transcription products were 180 nt for *rrnC* and 186 nt for *rrnE*, a major primer extension product corresponding to a transcript of ~138 nt was observed using a primer that should anneal to all of the *C. difficile rrn* operons (Fig. 5). In addition, several minor products that are likely to represent processing intermediates were also observed (Fig. 5).

The *gdh* promoter activates transcription of a *gusA* reporter fusion in *C. perfringens*. To test whether the *gdh* promoter region recognized in in vitro transcription reactions by *C. difficile* RNA polymerase and identified by primer extension analysis of cellular RNA does indeed serve as a functional promoter in living cells, the *gdh* promoter DNA was fused to the β-glucuronidase gene in plasmid pTUM177, and the resulting plasmid, pTUM270, was introduced into *C. perfringens* SM101. (*C. perfringens* was used as a surrogate host for this experiment because of the technical difficulty in introducing DNA into *C. difficile*). As shown in Fig. 6, the P*gdh-gusA* fusion gave a high level of β-glucuronidase activity in cells in TY medium. (We have assumed here that the start point of *gdh-gusA* expression is the same as that determined by primer extension for the intact *gdh* gene.) Synthesis of GusA was repressed by the presence of glucose (TYG medium), indicating that the *gdh* gene is subject to catabolite repression. Repression of gluta-

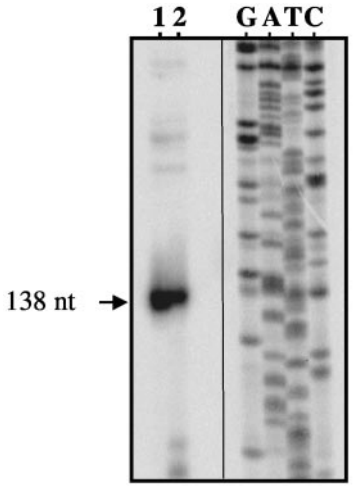

FIG. 5. Primer extension of *rrn* transcripts in vivo. Total RNA from *C. difficile* VPI 10463 and CD630 was used as a template to extend primer ONM61 using reverse transcriptase. The reverse-transcribed products of the *C. difficile* rRNA genes from VPI 10463 (lane 1) and CD630 (lane 2) were subjected to electrophoresis alongside DNA-sequencing reaction products generated using plasmid pTUM550 (harboring the *C. difficile rrnE* gene) as a template and primer ONM61 (shown on the right). The arrow points to a major primer extension product of 138 nt.

mate dehydrogenase gene transcription by glucose has also been reported for *B. subtilis* (3) and presumably reflects the fact that glutamate is a potential carbon source. *C. perfringens* carrying the promoterless vector pTUM177 showed no detectable β-glucuronidase activity.

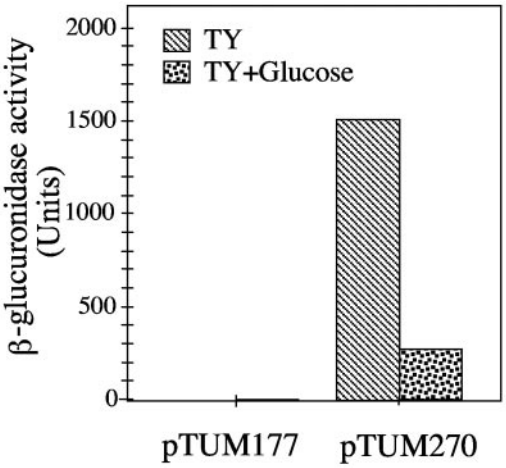

FIG. 6. Expression of a P*gdh-gusA* fusion in *C. perfringens*. A fragment of DNA carrying the *gdh* gene promoter was cloned into the reporter fusion vector pTUM177 and introduced into *C. perfringens* SM101. β-Glucuronidase activity of cells carrying either the promoterless fusion vector pTUM177 or the *gdh* promoter-containing plasmid pTUM270 (grown either in TY medium or in TY medium containing glucose) was assayed as described previously (9). Striped bars, TY medium; dotted bars, TYG medium.

DISCUSSION

We have partially purified RNA polymerase from *C. difficile* cells and used it to develop an in vitro transcription system that accurately initiates transcription from bona fide promoters of the *C. difficile* glutamate dehydrogenase (*gdh*) gene and rRNA gene (*rrnC* and *rrnE*) operons. The failure of *C. difficile* RNA polymerase to adhere to columns of heparin-Sepharose, single-stranded salmon sperm DNA-agarose, calf thymus DNA-cellulose, or Biorex-70 greatly impeded our ability to obtain a more highly purified enzyme. RNA polymerases from other sources generally bind to such columns. In fact, the use of heparin-agarose for the purification of *C. perfringens* RNA polymerase has been reported previously (10), and both heparin-agarose and single-stranded DNA-cellulose columns have been used to purify *Clostridium acetobutylicum* RNA polymerase (26). The inability of the *C. difficile* enzyme to adhere to these columns is unexplained.

Our preparation of *C. difficile* RNA polymerase was insufficiently pure to enable us to determine its sigma factor content. Nonetheless, the ability of this enzyme to initiate transcription accurately at three promoters that are expressed at high levels during a rapid exponential growth phase suggests that the enzyme had a significant amount of σ^A. We also know that our preparation of *C. difficile* RNA polymerase does not contain a significant amount of the alternative sigma factor TcdR, since this preparation was able to produce a transcript from the toxin promoters in vitro only when supplemented with purified TcdR (23).

Subregions 2.4 and 4.2 of sigma factors of the σ^{70} family (the predominant family of sigma factors) interact with the −10 and −35 sequences, respectively, of the promoter region. Since the subregions 2.4 and 4.2 of *C. difficile* σ^A are virtually identical to those of *B. subtilis* σ^A (30), it is not surprising that the *gdh* gene and *rrn* operons have promoter consensus sequences that are similar to those of *B. subtilis* σ^A-dependent promoters (14) and *E. coli* σ^{70}-dependent promoters (13) (Fig. 7). Furthermore, we noticed additional conserved features typical of gram-positive promoters that are thought to play a role in determining promoter strength, such as the dinucleotide TG at positions −15 and −14 and the presence of an A-rich region near position −43 (14) (Fig. 7). The ability of *E. coli* and *B. subtilis* RNA polymerase holoenzymes to bind to and initiate transcription from the same site as the *C. difficile* RNA polymerase strongly suggests similarity in the rules of promoter recognition among these bacteria. Moreover, an inspection of the likely promoter regions of 11 rRNA gene operons in the completed sequence of the *C. difficile* genome (http://www.sanger.ac.uk/Projects/C_difficile/) revealed that at least six other rRNA gene operons have promoter sequences highly similar to those of the *C. difficile rrnC* and *rrnE* promoters (Fig. 7) and share similarities with the σ^{70} promoters of *E. coli* rRNA genes (8).

Primer extension analysis of the *C. difficile gdh* gene revealed a promoter with a −10 region that showed perfect identity with that of the σ^{70} consensus sequence, but the −35 region showed poor conservation. This result might suggest the involvement of additional proteins in the expression of the *gdh* gene. When introduced into *C. perfringens*, expression of a DNA fragment containing this promoter region fused to a β-glucuronidase reporter was highly activated in the absence of glucose and was

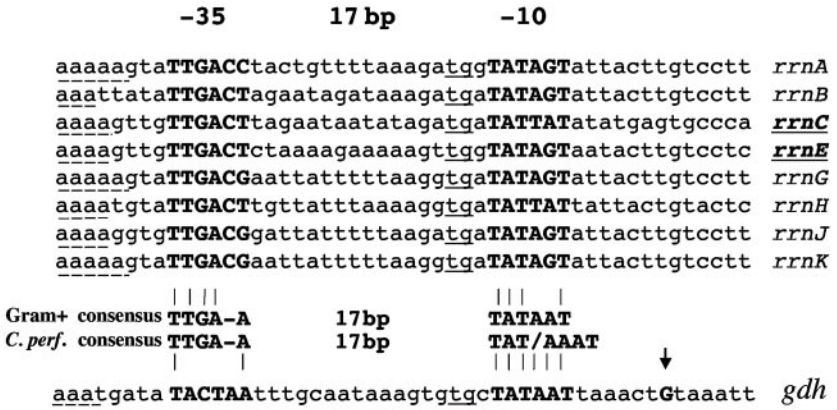

FIG. 7. Comparison of *gdh* and *rrn* promoters. A comparison of *C. difficile* promoters to the canonical gram-positive and *C. perfringens* consensus sequences (27) is shown. For the *rrnC*, *rrnE*, and *gdh* genes, the transcription start points utilized by major forms of RNA polymerase from *E. coli*, *B. subtilis*, and *C. difficile* were defined by in vitro runoff transcription assays (Fig. 3 to 5), whereas for *rrnA*, *rrnB*, *rrnG*, *rrnH*, *rrnJ*, and *rrnK*, the promoters were identified by homology to the *rrnC* and *rrnE* promoters. The names of the *rrn* operons were assigned based on their positions in the total genome sequence. Additional features typically found in gram-positive promoters, such as the dinucleotide TG at positions −15 and −14 and the A-rich region at position −43, are underlined with solid and dashed lines, respectively.

repressed in its presence. These results indicate that the *C. difficile gdh* promoter is recognized by the *C. perfringens* transcription machinery and is subject to carbon source-mediated regulation in the heterologous host.

The availability of *C. difficile* RNA polymerase preparations that faithfully recognize bona fide *C. difficile* promoters has enabled us to study the regulation of typical growth genes as well as toxin production in this bacterium (23). Such approaches can now be applied to the elucidation of other gene expression mechanisms in this important human pathogen.

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REFERENCES

1. **Apirion, D., and A. Miczak.** 1993. RNA processing in prokaryotic cells. Bioessays **15:**113–120.
2. **Banno, Y., T. Kobayashi, H. Kono, K. Watanabe, K. Ueno, and Y. Nozawa.** 1984. Biochemical characterization and biologic actions of two toxins (D-1 and D-2) from *Clostridium difficile.* Rev. Infect. Dis. **6:**S11–S20.
3. **Belitsky, B. R., and A. L. Sonenshein.** 1998. Role and regulation of *Bacillus subtilis* glutamate dehydrogenase genes. J. Bacteriol. **180:**6298–6305.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
5. **Braun, V., T. Hundsberger, P. Leukel, M. Sauerborn, and C. von Eichel Streiber.** 1996. Definition of the single integration site of the pathogenicity locus in *Clostridium difficile.* Gene **181:**29–38.
6. **Burgess, R. R., and J. J. Jendrisak.** 1975. A procedure for the rapid, large-scale purification of *Escherichia coli* DNA-dependent RNA polymerase involving polymin P precipitation and DNA-cellulose chromatography. Biochemistry **14:**4634–4638.
7. **Cohen, S. H., Y. J. Tang, and J. Silva, Jr.** 2000. Analysis of the pathogenicity locus in *Clostridium difficile* strains. J. Infect. Dis. **181:**659–663.
8. **Condon, C., C. Squires, and C. L. Squires.** 1995. Control of rRNA transcription in *Escherichia coli.* Microbiol. Rev. **59:**623–645.
9. **Dupuy, B., and A. L. Sonenshein.** 1998. Regulated transcription of *Clostridium difficile* toxin genes. Mol. Microbiol. **27:**107–120.
10. **Garnier, T., and S. T. Cole.** 1988. Studies of UV-inducible promoters from *Clostridium perfringens in vivo* and *in vitro.* Mol. Microbiol. **2:**607–614.
11. **George, R. H., J. M. Symonds, F. Dimock, J. D. Brown, Y. Arabi, N. Shinagawa, M. R. B. Keighly, L. Alexander-Williams, and D. W. Burdon.** 1978. Identification of *Clostridium difficile* as a cause of pseudomembranous colitis. Br. Med. J. **1:**695.
12. **Hammond, G. A., and J. L. Johnson.** 1995. The toxigenic element of *Clostridium difficile* strain 10463. Microb. Pathog. **19:**203–213.
13. **Hawley, D. K., and W. R. McClure.** 1983. Compilation and analysis of *Escherichia coli* promoter DNA sequences. Nucleic Acids Res. **11:**2237–2255.
14. **Helmann, J. D.** 1995. Compilation and analysis of *Bacillus subtilis* σ^A-dependent promoter sequences: evidence for extended contact between RNA polymerase and upstream promoter DNA. Nucleic Acids Res. **23:**2351–2360.
15. **Herbert, M., T. A. O'Keeffe, D. Purdy, M. Elmore, and N. P. Minton.** 2003. Gene transfer into *Clostridium difficile* CD630 and characterisation of its methylase genes. FEMS Microbiol. Lett. **229:**103–110.
16. **Just, I., J. Selzer, M. Wilm, C. von Eichel-Streiber, M. Mann, and K. Aktories.** 1995. Glucosylation of Rho proteins by *Clostridium difficile* toxin B. Nature **375:**500–503.
17. **Just, I., M. Wilm, J. Selzer, G. Rex, C. von Eichel-Streiber, M. Mann, and K. Aktories.** 1995. The enterotoxin from *Clostridium difficile* (ToxA) monoglucosylates the Rho proteins. J. Biol. Chem. **270:**13932–13936.
18. **Karlsson, S., B. Dupuy, K. Mukherjee, E. Norin, L. G. Burman, and T. Åkerlund.** 2003. Expression of *Clostridium difficile* toxins A and B and their sigma factor TcdD is controlled by temperature. Infect. Immun. **71:**1784–1793.
19. **Kelly, C. P., and J. T. LaMont.** 1998. *Clostridium difficile* infection. Annu. Rev. Med. **49:**375–390.
20. **Larson, H. E., and A. B. Price.** 1977. Pseudomembranous colitis: presence of clostridial toxin. Lancet **ii:**1312–1314.
21. **Losick, R., R. G. Shorenstein, and A. L. Sonenshein.** 1970. Structural alteration of RNA polymerase during sporulation. Nature **227:**910–913.
22. **Lowe, P. A., D. A. Hager, and R. R. Burgess.** 1979. Purification and properties of the σ subunit of *Escherichia coli* DNA-dependent RNA polymerase. Biochemistry **18:**1344–1352.
23. **Mani, N., and B. Dupuy.** 2001. Regulation of toxin synthesis in *Clostridium difficile* by an alternative RNA polymerase sigma factor. Proc. Natl. Acad. Sci. USA **98:**5844–5849.
24. **Mani, N., D. Lyras, L. Barosso, P. Howarth, T. Wilkins, J. I. Rood, A. L. Sonenshein, and B. Dupuy.** 2002. Environmental response and autoregulation of *Clostridium difficile* TxeR, a sigma factor for toxin gene expression. J. Bacteriol. **184:**5971–5978.
25. **Moncrief, J. S., D. M. Lyerly, and T. D. Wilkins.** 1999. Molecular biology of large clostridial toxins, p. 333–359. *In* K. Atories and I. Just (ed.), Bacterial protein toxins. Handbook of experimental pharmacology, vol. 145. Springer-Verlag, Berlin, Germany.
26. **Pich, A., and H. Bahl.** 1991. Purification and characterization of the DNA-

dependent RNA polymerase from *Clostridium acetobutylicum.* J. Bacteriol. **173:**2120–2124.

27. **Rood, J. I., and S. T. Cole.** 1991. Molecular genetics and pathogenesis of *Clostridium perfringens.* Microbiol. Rev. **55:**621–648.
28. **Rupnik, M., B. Dupuy, N. F. Fairweather, D. N. Gerding, S. Johnson, I. Just, D. M. Lyerly, M. R. Popoff, J. I. Rood, A. L. Sonenshein, M. Thelestam, B. W. Wren, T. D. Wilkins, and C. von Eichel-Streiber.** 2005. Revised nomenclature of *Clostridium difficile* toxins and associated genes. J. Med. Microbiol. **54:**113–117.
29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
30. **Sonenshein, A. L., J. D. Haraldsen, and B. Dupuy.** 2005. RNA polymerase and alternative σ factors, p. 607–629. *In* P. Dürre (ed.), Handbook on clostridia. CRC Press, Boca Raton, Fla.
31. **Sullivan, N. M., S. Pellett, and T. D. Wilkins.** 1982. Purification and characterization of toxins A and B of *Clostridium difficile.* Infect. Immun. **35:** 1032–1040.
32. **von Eichel-Streiber, C., P. Bouquet, M. Sauerborn, and M. Thelestam.** 1996. Large clostridial cytotoxins—a family of glucosyltransferases modifying small GTP-binding proteins. Trends Microbiol. **4:**375–382.
33. **Whipple, F. W., and A. L. Sonenshein.** 1992. Mechanism of initiation of transcription by *Bacillus subtilis* RNA polymerase at several promoters. J. Mol. Biol. **223:**399–414.
34. **Zhao, Y., and S. B. Melville.** 1998. Identification and characterization of sporulation-dependent promoters upstream of the enterotoxin gene (*cpe*) of *Clostridium perfringens.* J. Bacteriol. **180:**136–142.